Appendix F
(F1-F3)

Appendix F1

Northern Blot Analysis Protocol

1. SCHWANN CELL PREPARATION

2L of neonate rat pups (Sprague Dawley) (at Post-pardum day 2-Post-pardum day 3 stage) were placed on ice to euthanize. Pups were then removed and decapitated to drain the blood. The neonates were placed, belly-down, on a dissection board and rinsed with 70% ethanol to sterilize. Using a scalpel, the skin was removed in the thigh area until the sciatic nerve was exposed (or until a thin white "string" extended from the spinal cord to the knee was visible). The nerves were placed in DMEM medium and then aspirated, followed by bringing the volume to 2.4 ml with DMEM media and adding 300uL 10X Collagenase (0.3%, Sigma Cat. #C-9891) and 300uL 10X Trypsin (.25%, GIBCO Cat. #25095-019) for dissociation. Nerves were then incubated at 37°C for 15 min, centrifuged for 5 min at 1,000 rpm followed by removing the media (repeated twice). 1 mL DMEM-HEPES and 1mL DMEM/10% FBS were added and then transfered to a 50mL conical tube. The contents of the tube were sheared with the following gauge needles (VWR): once with 18G, twice with 21G and twice with 23G. The contents were placed on a Falcon cell strainer and spun at a very low speed (about 1200 rpm). The total volume was brought to 10mL with DMEM/10% FBS and plated on a Poly-L-lysine treated 10cm plate (Sigma, Cat. #P-1274). Plates were then incubated overnight in 37°C humid incubator at 7% CO2. Fresh media added with 100X ARA C (10mM, Sigma, Cat. #C-1768) and cultured for an additional 48 hours. The cells were then washed with PBS (three times) to remove the ARA C and the following were added: DMEM/10% FBS, different concentrations of Forskolin in 100% ethanol (2uM, 5uM, 10uM, 20uM and 50uM) (Calbiochem, Cat#344270), 80ug of Pituitary Extract (Sigma, #P-1167) in PBS and 0.1%BSA, followed by growing the cells for 30 hours at 37°C humidifier at 7% CO_2. The cells were then collected and the RNA was isolated and analyzed.

Antibody selection was accomplished according to the following: the Poly-L-Lysine treated plates were first washed with 1X PBS (three times), trypsinized with 1mL of 0.5% trypsin-EDTA, for about 1 min and then neutralized with 9mL of DMEM-HEPES buffer and 10% FBS. Cells were centrifuged at 1200rpm for 5 min, resuspended in 3mL of DMEM-HEPES to wash out the trypsin and spun for 5 min at 1200rpm. Cells were then resuspended in 600uL of DMEM-HEPES, leaving some media after the spin in order to have single cells. Thy1.1 antibody (Monoclonal Antibody, Sigma, Cat. #P-1274) was added at a 1:1000 dilution.

Appendix F1 cont'd

The cells were then incubated for 20 min at 37°C, slightly agitating the tube every two minutes. 20uL of Guinea Pig complement (GIBCO, Cat. #19195-015) was thawed before using it, followed by adding the complement to the cells with the antibody to a final volume of 1mL. The cells were incubated for about 20 min-30 min at 37°C water bath and 10mL of DMEM-HEPES was added and spun down for 5 min at 1200rpm. Cells were resuspended in 5mLs of DMEM/10% FBS and added to poly-L-lysine treated plates that contains pituitary extract and forskolin. The cells were left to recover for 24-48 hours and the immune selection procedure was repeated twice.

2. PREPARATION OF CRUSHED RAT SCIATIC NERVE

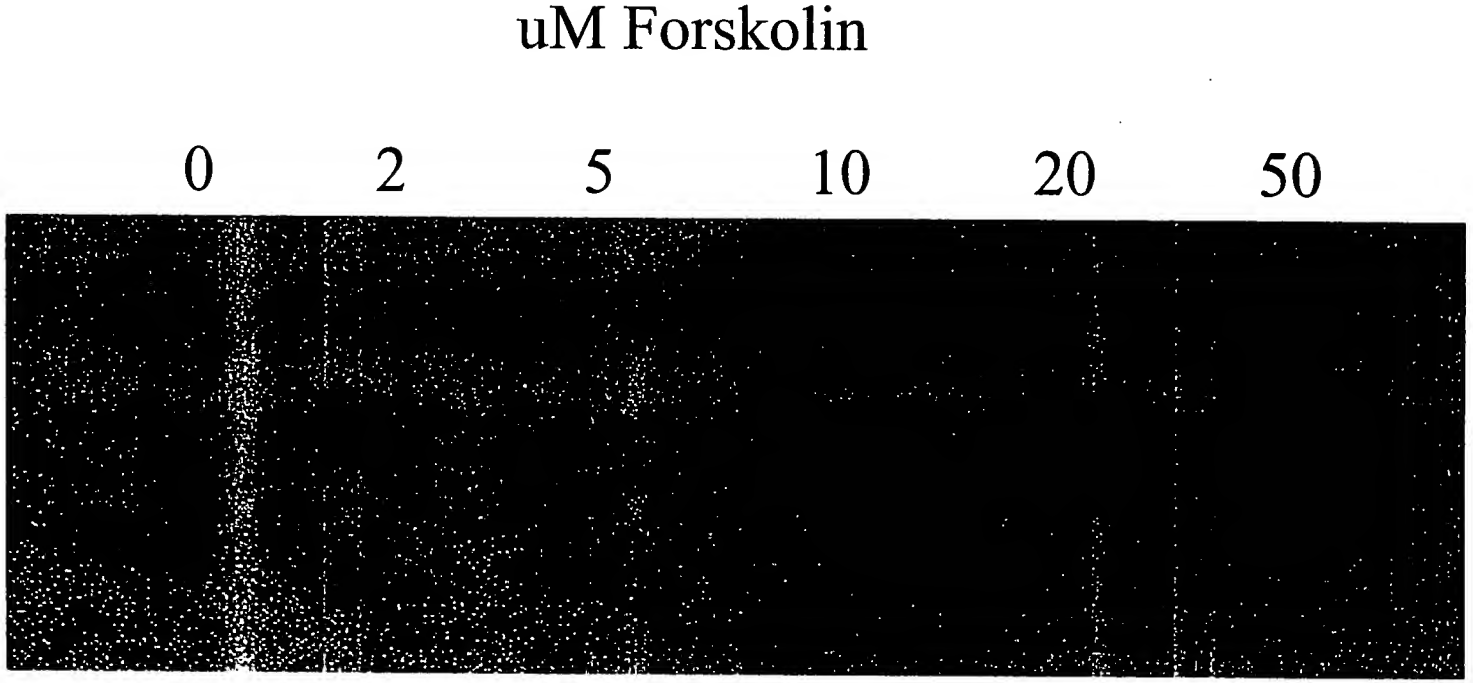
The sciatic nerves of anesthetized (iso-florene), adult (10-13 week old) Sprague-Dawley rats were exposed at the sciatic notch. Nerve crush was produced by tightly compressing the sciatic nerve at the sciatic notch with flattened forceps twice, each time for 10 sec; this technique causes all the axons to degenerate, but allows axonal regeneration. At varying times after nerve injury, the animals were euthanized by CO_2 inhalation, the distal nerve stumps were removed, and the most proximal 2-3 mm was trimmed off. For crushed nerves, the entire distal nerve was harvested. The nerves were immediately frozen in liquid nitrogen and stored at –80°C. Unlesioned sciatic nerves were obtained from animals of varying ages, from P0 (post crush) to P13.

3. NORTHERN ANALYSIS

RNA from the schwann cell preparation was harvested utilizing RNAzol B reagent (TelTest Inc., Cat. #CS-104), according to manufacturer's instructions. After electrophoresis in an 1% agarose/formaldehyde gel, the RNA was transferred to a nylon membrane (Sachleicher Schull) by capillary action using 10X SSC. A ^{32}P-labelled 19M DNA probe was synthesized using a DNA fragment corresponding precisely to the 3' end of 19M and a High Prime labeling kit (Roche Molecular Biochemical) according to the manufacturer's instructions. Hybridization was performed using ExpressHyb Solution (Clontech, Cat. #8015-2) supplemented with 100µg/ml salmon sperm DNA as follows. The membrane containing the separated RNA samples was first incubated with ExpressHyb solution at 65°C overnight. The ^{32}P-labelled 19M DNA probe was denatured by boiling for 2 minutes, placed on ice for 5 minutes and then transferred into the ExpressHyb solution bathing the membrane. After an overnight incubation at 65°C, the membrane was removed from the hybridization solution and washed four times for 15 minutes each in 2XSSC/1% SDS at 65°C, followed by two washes for 15 minutes each in 0.2XSSC/0.1% SDS at 55°C. Excess moisture was

removed from the blot by gentle shaking, after which the blot was wrapped in plastic wrap and exposed to film overnight at –80°C.

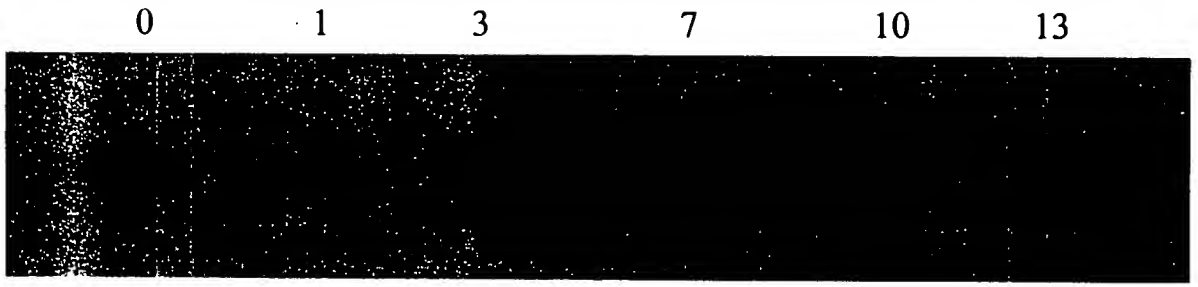
Northern Analysis of 19M in Forskolin Treated Rat Schwann Cells



Appendix F2

Northern Analysis of 19M Expression in Crushed Rat Sciatic Nerve

Days post-crush



Appendix F3